Triparticalcar equi is a new coprophilous species within Spizellomycetales, Chytridiomycota

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ABSTRACT

Herbivore dung is a well-known habitat for filamentous fungi; however, we are only beginning to examine this substrate for chytridiomycete diversity. During a recent survey of chytrids of Tuscaloosa County, Alabama, we isolated a novel species in the genus *Triparticalcar* from herbivore dung. We examined two strains (WJD101, WJD156) with light and transmission electron microscopy and compared them to the type species, *T. articum*. We also analyzed partial nuc 28S rDNA D1-D2 domains (28S) and nuc rDNA regions encompassing the internal transcribed spacers 1 and 2 and 5.8S (ITS) to determine the phylogenetic placement of the strains within a broader sampling of Spizellomycetales. Our molecular phylogeny confirmed that the two strains belong to a new phylogenetic species within *Triparticalcar*. The two isolates are distinguishable from the type by the development and morphology of their rhizoids, and we describe the new species as *T. equi*, the first formal description of a new species in this genus since it was erected. Published on-line www.phytologia.org *Phytologia 98(4): 241-249 (Oct 6, 2016)*. ISSN 030319430.

KEY WORDS: *Triparticalcar equi* **sp. nov.**, chytrid, dung, molecular phylogeny, Spizellomycetales, taxonomy, zoospore ultrastructure.

Herbivore dung is a well-known habitat for ascomycetes, basidiomycetes, and fungi formerly classified as zygomycetes (Bell, 1983). However, the diversity of chytrid fungi (Chytridiomycota) inhabiting dung is under explored. Wakefield et al. (2010) first reported strains representing the Spizellomycetales genera *Gaertneriomyces* D. J. S. Barr and *Triparticalcar* D. J. S. Barr from dung, and some of these dung strains were molecularly divergent from existing species of the genera and probably are novel taxa. Expanding the list of dung chytrids within the Spizellomycetales, Simmons (2011) reported the genus *Geranomyces* D. R. Simmons from dung, and Simmons and Longcore (2012) described a new genus, *Fimicolochytrium* D. R. Simmons & Longcore, based on a species isolated from dung. A new member of Lobulomycetales, *Alogomyces tanneri* D. R. Simmons and Letcher, was also isolated from dung (Simmons et al., 2012). Simmons (2012) examined biodiversity within herbivore dung with next-generation sequencing and found it rich in sequences that form a well-supported clade with *Triparticalcar arcticum* (D. J. S. Barr) D. J. S. Barr.

Barr (1970) isolated *Triparticalcar arcticum*, the type and currently only species of *Triparticalcar* (Barr, 1980), from Canadian high arctic saline clay soil and originally placed it in the genus *Phlyctochytrium* J. Schröt based on thallus morphology and development. Isolated from pollen, it had one to many papillae, a globose or peg-like apophysis, and spherical zoospores with the ability to become amoeboid. Based on its distinct zoospore ultrastructure (Barr and Allan, 1981; Chong and Barr, 1973), including a tripartite spur, anterior position of the nucleus and lipid globules, and posterior placement of mitochondria, Barr (1980) placed the species into the new genus *Triparticalcar*. The molecular phylogeny of Wakefield et al. (2010) validated the monophyly of *Triparticalcar* and its divergence from other spizellomycetalean genera.

During an investigation of chytrid diversity in Tuscaloosa County, Alabama, we baited horse and cow dung samples for chytrids. The resulting strains, designated WJD 101 and WJD 156, were morphologically similar to *T. arcticum*, and in a molecular phylogeny based on partial nuc 28S rDNA D1-D2 domains (28S), the two strains grouped sister to *T. arcticum* (Davis et al., 2013). Davis et al.

(2013) suggested that the strains represented a new species within the genus; however, their analysis did not include other members of the *Triparticalcar* clade from Wakefield et al. (2010) nor was a morphological description included. Herein, we infer a new molecular phylogeny that includes both the new isolates and members of the *Triparticalcar* clade from Wakefield et al (2010) and compare morphology of cultures WJD 101 and WJD 156 with that of the extype culture of *Triparticalcar arcticum* (strain BR 59). Based on the results, we describe the new species *Triparticalcar equi.*

MATERIALS AND METHODS

Isolation and culture: Dr. Pete Letcher collected cow dung from a farm in Duncanville, Alabama, and Dr. Carol Duffy collected horse dung from Timber Acres Ranch in Buhl, Tuscaloosa County, Alabama. A subsample of each dung collection was placed in a sterile Petri dish, flooded with sterile water, and baited with pine pollen (Davis et al., 2013). Strain WJD 101 was cultured from cow dung and strain WJD 156 from horse dung. Strain BR 059 was obtained from the American Type Culture Collection (ATCC #18785). All cultures were maintained on PmTG (Barr, 1987; 1 g peptonized milk, 1 g tryptone, 5 g glucose, 10 g agar, 1 L double-distilled water) nutrient agar in Parafilm-sealed Petri dishes at room temperature (20–25°C) in the dark.

DNA extraction and amplification: We extracted genomic DNA as described in Davis et al. (2013) and amplified the nuc rDNA region encompassing the internal transcribed spacers 1 and 2 and 5.8S (ITS) with the ITS4/ITS5 primer pair (White et al., 1990). PCR conditions and cycles were those used in Davis et al. (2013). Amplicons were sequenced by Macrogen Corp USA (Rockville MD), and we assembled sequenced amplicons into contiguous sequences using default settings in Sequencher 4.5 (Genecodes).

Phylogenetic analysis: We downloaded the 28S sequences of WJD 101 and WJD 156 (Davis et al., 2013) and 17 additional members of Spizellomycetales (Simmons, 2011; Wakefield et al., 2010) from GenBank and aligned them with ClustalX (Thompson et al., 1997) followed by manual adjustment in BioEdit (Hall, 1999). The alignment was deposited in TreeBase http://purl.org/phylo/treebase/phylows/study/TB2:S190 96. We used PAUPRat (Sike and Lewis, 2001) to infer maximum parsimony (MP) trees and generated support values as heuristic searches with 500 replicates, each with 10 random-addition replicates. We determined the best-fit model of nucleotide substitution with MrModeltest 2.3 (http://www.abc.se/nylander) and inferred Maximum Likelihood (ML) trees with GARLI 0.951 (Zwikl, 2006). Branch support was assessed with 500 bootstrapping replicates. The inferred trees included 19 spizellomycetalean members and were rooted with two members of the sister order Rhizophlyctidales. Molecular divergence among strains WJD 101, WJD 156, and BR 059 in the 28S and ITS rDNA regions were determined by pair-wise similarity comparisons in BioEdit (Hall, 1999).

Morphology: We inoculated nutrient agar plates (PmTG) with zoospore suspensions of the three strains and recorded morphology of different developmental stages with brightfield and phase contrast light microscopy (Zeiss Axioskop with a Zeiss AxioCam MRc3 camera). Mature sporangia were stained with 0.1% toluidine blue to observe morphology of discharge papillae (Letcher et al., 2015; Parker et al., 1982).

Zoospore ultrastructure: Zoospores were collected and fixed for examination with a Hitachi 7650 transmission electron microscope (TEM). Three- to four-day old plates of WJD 156 and WJD 101 were flooded with sterile water to initiate zoospore discharge. Zoospores were collected in 15 min intervals and fixed in 2.5% glutaraldehyde in 0.1 M *s*-collidine buffer overnight at 20 C. Fixation was continued in1% osmium tetroxide in 0.1M *s*-collidine buffer overnight in the dark at 20 C. Centrifugation at 3 *g* was used to produce a pellet of fixed zoospores, which was then embedded in molten agar. The pellet was trimmed into 1 mm × 1mm blocks and suspended in saturated aqueous uranyl acetate overnight in the dark on a

shaker. The blocks were dehydrated in a graded acetone series (10%, 30%, 50%, 70%, 85%, 95%, 100%, 100%) held for 15 min at each step. A graded series was used to infiltrate the blocks with EPON resin: 12% for 1 h, 25% for 4 h, 50% for 4 h, 75% for 12 h, 100% for 24 h, and 100% for 24 h. The EPON was polymerized for 3 days at 72 C. Sections of 100 nm thickness were obtained using a diamond knife on a Leica Ultracut microtome and were collected on 300-mesh hexagonal nickel grids. Grids with sections were treated with 1% periodic acid (4 min) to enhance staining and post-stained with uranyl acetate in 70% ethanol (10 min) and lead citrate in the presence of sodium hydroxide (6 min). Sections were observed at 60 kV with a Hitachi 7650 transmission electron microscope (TEM).

RESULTS

Phylogenetic analysis: The 28S sequences of WJD 156 and WJD 101 were 99% similar to each other and 90% similar to *Triparticalcar arcticum* BR 059. Internal transcribed spacer sequences of WJD 156 and WJD 101 were 89% similar to each other and 57% similar to *T. arcticum* BR 059, which made them unalignable even within the genus; thus, ITS was excluded from tree inference. The alignment of 28S sequences contained 861 characters of which 315 were parsimony informative. From 1005 PAUPRat inferred trees, 990 were equally parsimonious (L= 1370 steps, CI= 0.393, RI= 0.711). The best model of base pair substitution was GTR + G. MP and ML (-lnL = 4212.74) trees were congruent and only the ML tree is depicted here (FIG.1). Figure 1 shows that isolates WJD 156 and WJD 101 form a well-supported (100% bootstrap support) sister clade to *T. arcticum* (BR 059, JEL 554, JEL 555, JEL 560). The relationships between the *Triparticalcar* clade and other members of Spizellomycetales are not fully resolved in this analysis (FIG. 1).

Morphology: The development and morphology of *T. arcticum* are treated extensively elsewhere (Barr 1970, 1984) and will be treated briefly here. Descriptions will follow the terminology of Barr (1984). On agar, BR 059 germlings have a broad, peg-like rhizoidal axis (FIG. 2A) with rhizoids typically developing at the end of the rhizoidal axis and occasionally developing laterally (FIG. 2A, B). Rhizoids are generally stout, branch terminally, and taper evenly into blunt ends (FIG. 2B). At maturity, the main rhizoidal axis becomes bulbous or a multibranched process (FIG. 2C). Rhizoids form a short, dense network (FIG. 2C), and zoosporangia produce two to three discharge papillae (FIG. 2D).

WJD 156 and WJD 101 followed similar developmental pathways and share the same morphological features. Hence, observations made on both are reported together, and only images of WJD156 are shown. On nutrient agar, zoospore cysts form narrow, long rhizoidal axes (FIG. 2E), and rhizoids develop laterally and terminally (FIG. 2F). Through development, rhizoids are long, isodiametric, and blunt-ended with lateral and terminal branches, and the rhizoidal axis is isodiametric initially and becomes bulbous or inflated in shape (FIG. 2F). At maturity, the rhizoidal system is dense with long, extensively branched rhizoids that obscure the rhizoidal axis (FIG. 2G). Mature zoosporangia are covered with numerous cylindrical, protruding discharge papillae approximately 2-5μm high (FIG. 2H).

Zoospore ultrastructure: The nucleus and several lipid globules are anteriorly positioned (FIG. 3A). In longitudinal section, mitochondria are predominantly posteriorly positioned (FIG. 3A), and in transverse section, mitochondria are also observed anteriorly positioned (FIG. 3B). Microbodies are associated with the lipid globules with mitochondria located close by (FIG. 3B). Ribosomes are dispersed (FIG. 3A, B). A tripartite spur and microtubule complex extend from the kinetosome to the nucleus (FIG. 3A, D, E). The posterior of the non-flagellated centriole is angled approximately 60° away from the kinetosome, and the cell membrane is invaginated near the flagellum (FIG. 3A, C).

TAXONOMY

Triparticalcar equi W. J. Davis, Letcher, & M. J. Powell **sp. nov.** FIGS. 2–3
MycoBank MB 816514

Typification: USA. ALABAMA: Tuscaloosa County, Buhl, Timber Acres Ranch, 33.227526, -87.739037. Pine pollen, horse dung, October 2010, *W. J. Davis 156* (**holotype** FIG. 2G, in Davis et al. 2016. Phytologia 98: 248).
Ex-type strain WJD 156 (UACCC); GenBank KC691398 (28S) and KX019807 (ITS).

Etymology: The specific epithet is the genitive noun of the Latin word for horse, *equus*, and the source of the dung that yielded the holotype culture.

Description: On nutrient agar, germlings have slender, long germ tubes; rhizoids develop laterally and terminally. Rhizoids are long with lateral and terminal branches, isodiametric with blunt ends, and dense at maturity. Rhizoidal axis is bulbous or inflated in shape and obscured at maturity. Mature zoosporangia have numerous discharge papillae 2-5μm high. Zoospores have a *Triparticalcar*-type ultrastructure.

Additional specimens examined: USA. ALABAMA: Tuscaloosa County, Duncanville, a farm. Pine pollen, cow dung, October 2010, *W. J. Davis 101*(UACCC WJD101); GenBank KC788571 (28S) and KX019806 (ITS).

DISCUSSION

Triparticalcar was first erected to accommodate a species isolated on pine pollen from high arctic soils (Barr, 1970, 1980). Subsequently, a number of phylogenetically divergent strains have been observed in this genus but not yet formally described (Davis et al., 2013; Simmons, 2012; Wakefield et al., 2010). Thus, we formally describe and name a new member of this genus, *T. equi*, a species firmly established as a member of this genus molecularly (FIG. 1) and ultrastructurally by the presence of the tripartite spur extending from the kinetosome to the nucleus (FIG. 3A, D, E).

Triparticalcar equi can be distinguished from the type *T. arcticum*. Developmentally, *T. equi* germlings have a narrower and longer germ tube than *T. arcticum* and rarely appear carrot or turnip shaped. In *T. equi* rhizoids develop laterally and terminally whereas in *T. arcticum* the rhizoids develop from the base of the germ tube. The rhizoids of *T. equi* are longer than those of *T. arcticum*, both during development and at maturity. Branching of rhizoids tends to occur laterally and terminally in *T. equi* rather than primarily terminally as in *T. arcticum*.

Molecularly, *T. arcticum* and *T. equi* are 10% divergent in the 28S rDNA region, which is comparable to other species in Chytridiomycetes. For example, in the Lobulomycetales, *Lobulomyces poculatus* (Willoughby & Townley) D. R. Simmons and *L. angularis* (Longcore) D. R. Simmons are 11% divergent in the 28S rDNA region (Simmons et al., 2009). In the Polychytriales, *Arkaya lepida* Longcore & D.R. Simmons and *A. serpentine* (Dogma) Longcore & D. R. Simmons are 7% divergent (Longcore and Simmons, 2012). In Chytridiales, the described species *Pseudorhizidium endosporangiatum* M. J. Powell, Letcher & Longcore is 8% divergent from an undescribed species in the genus (Letcher and Powell, 2014; Powell et al., 2013). In the Rhizophydiales, *Rhizophydium globosum* (A. Braun) Rabenh. and *R. brooksianum* Longcore are 7% divergent (Letcher et al., 2006).

Since this is the first species of *Triparticalcar* described since the genus was established with the single species, *T. arcticum*, our results highlight the undescribed diversity in this group (Barr, 1980). It is interesting that strains JEL554, JEL 555, and JEL 560 that are in the *T. arcticum* clade were also isolated on horse and cow manure (Wakefield et al., 2010). Strains JEL 250, JEL 355, PL 162 formed a clade sister of the *Triparticalcar* clade in our study (labeled unidentified sp.) but were included in the

Triparticalcar clade in Wakefield et al. (2010). Preliminary ultrastructural investigations indicate this sister clade is a new genus. Thus, our study demonstrates that as *Triparticalcar* is currently circumscribed with two species, *T. articum* and *T. equi*, it is a monophyletic genus.

Dung is a well-established source of basidiomycetes, ascomycetes, and fungi formerly classified as zygomycetes (Bell, 1983). However, we are in the early stages of observing the diversity of chytrid fungi that inhabit dung (Wakefield et al., 2010). Much of the diversity observed thus far can be placed in the Spizellomycetales; however, as exemplified by *Alogomyces tanneri*, additional orders may be present as well. More diversity awaits discovery and classification.

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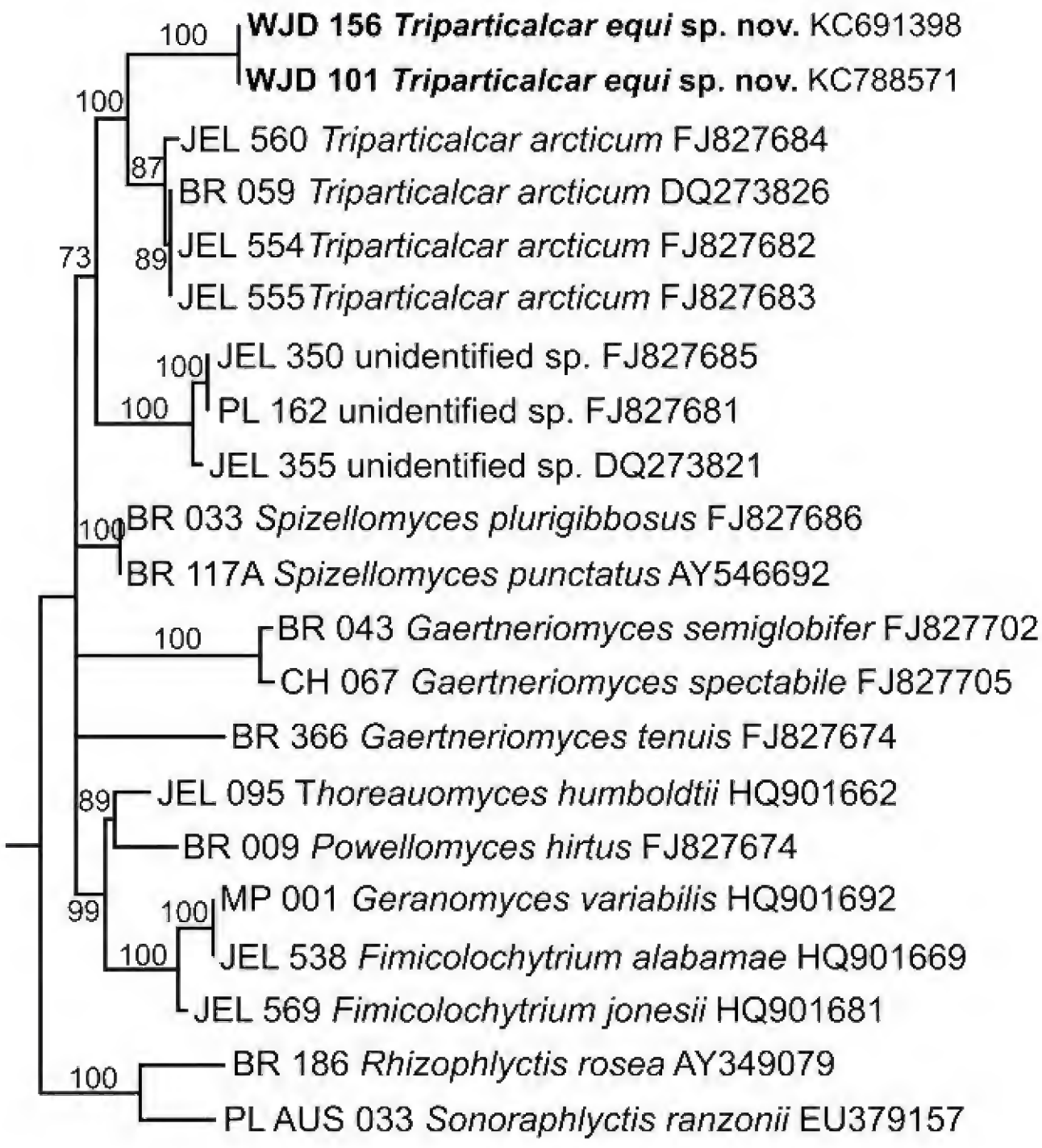

FIG. 1. Molecular phylogeny of 19 taxa in Spizellomycetales inferred from analyses of partial 28S rRNA gene sequences, with two members of Rhizophlyctidales as outgroup. Numbers above branches are ML bootstrap support values; –lnL = 4212.74

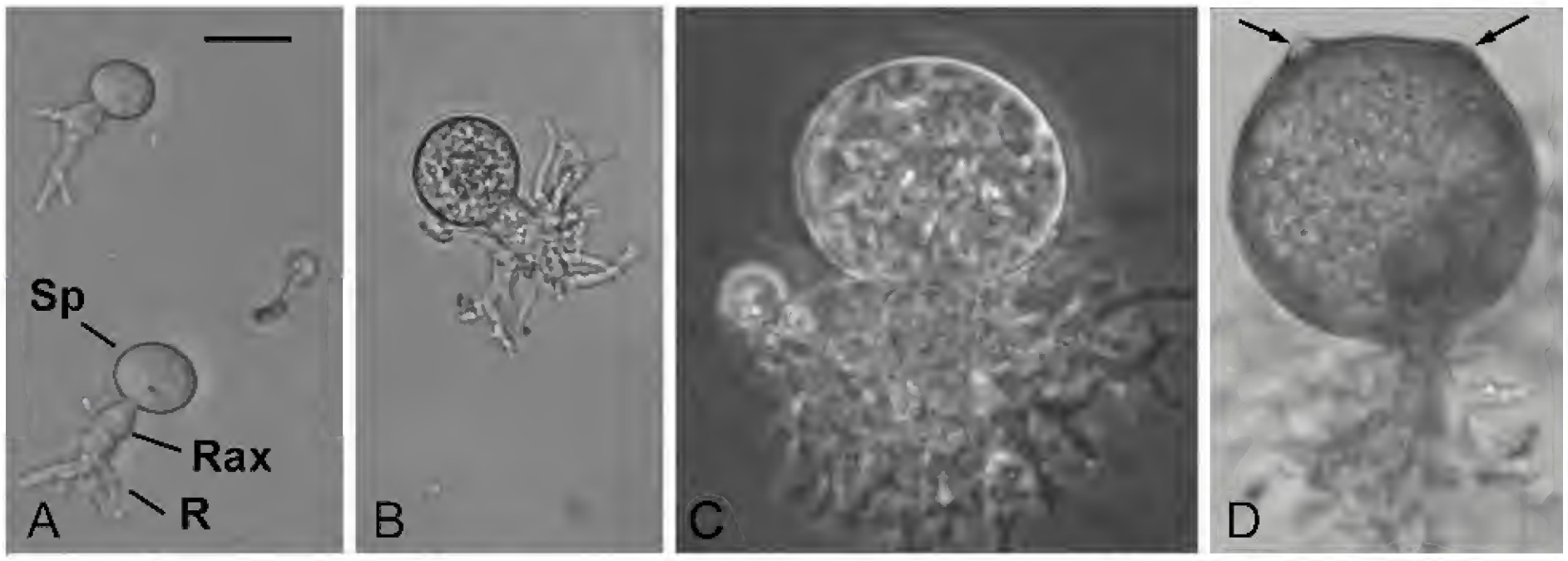

FIG. 2. Light micrographs of *Triparticalcar arcticum* BR 059(A-D) and *T. equi* WJD 156 (E-G) on nutrient agar. A-D. *T. arcticum*, BR 059, type. A. Germlings with stout rhizoidal axis and stubby rhizoids. B. Immature thallus with terminal branching of rhizoids. C. Maturing thallus with compact mass of stout, short rhizoids. D. Mature sporangium with two discharge papillae (arrows) stained with toluidine blue. E. Germinating zoospore with long, narrow rhizoidal axis. F. Immature thallus with stout rhizoidal axis and terminal and laterally branching rhizoids. G. Maturing thallus with compact mass of long, slender rhizoids. H. Mature sporangium with multiple discharge papillae (arrows) stained with toluidine blue. Abbreviations: Sp, sporangium; R, rhizoids; Rax, rhizoidal axis. Scale bar = 10 µm (A, B, E), 20 µm (C, D, F, G).

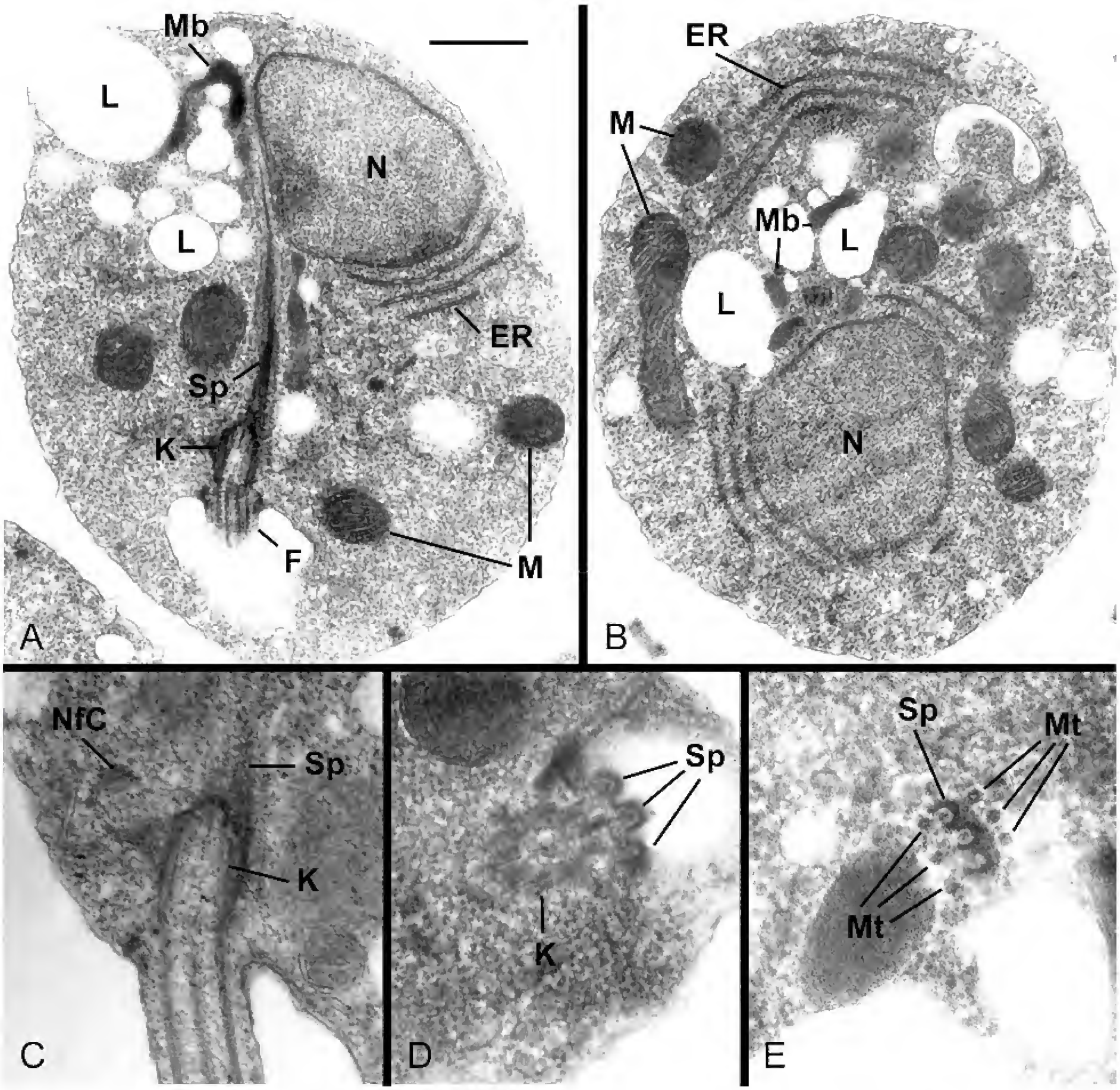

FIG. 3. Ultrastructural features of zoospore of *Triparticalcar equi.* A. Longitudinal section (LS), with an elongate spur originating at the kinetosome and extending anteriorly, microbodies interspersed among multiple lipid-globules, nucleus backed by multiple layers of endoplasmic reticulum, and multiple mitochondria. Ribosomes dispersed in the cytoplasm. B. Transverse section (TS). C. LS illustrating angle (~60°) between kinetosome and non-flagellated centriole. D. TS of kinetosome and spur. E. TS through spur and microtubules, approximately midway through zoospore body. Abbreviations: ER, endoplasmic reticulum; F, flagellum; K, kinetosome; L, lipid globules; M, mitochondria; Mb, microbody; Mt, microtubules; N, nucleus; NfC, non-flagellated centriole; Sp, spur. Scale bar in A = 0.5 µm (A, B), 0.25 µm (C, D), 0.2 µm (E).